AN ECONOMICAL INTRACUTANEOUS METHOD FOR TESTING THE VIRULENCE OF DIPHTHERIA BACILLI *

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The testing of the virulence of isolated strains of diphtheria and diphtheria-like bacilli is important in particular for the following reasons:

1. Four to eight percent of individuals in localities where diphtheria is endemic, are carriers of bacilli morphologically and culturally like the diphtheria bacillus, but non-virulent in 30-50% of cases. At the Willard Parker Hospital in New York, Wilcox and Taylor found that 4.5% of the cases admitted to the scarlet fever wards were bacillus carriers, and that of the isolated organisms only one half were virulent.

2. According to Neisser, individuals who become persistent carriers after an attack of diphtheria show only non-virulent forms in fully 20% of cases. The absence of virulence in the organisms isolated from these carriers is probably permanent, since in our opinion these bacilli are not derived from the virulent bacilli which excited the disease, but rather from bacilli which were originally non-virulent. Therefore, if this fact were established, such individuals could be discharged from quarantine.

Morphologically, the non-virulent strains cannot be separated from the virulent, and in their sugar reactions the two types are in a majority of instances similar. Hence, the animal test must be employed for final diagnosis in doubtful cases. The method thus far in vogue for testing the virulence of diphtheria bacilli has been to isolate the strain, grow it for 48 hours in ascitic broth (1 part ascitic fluid and 2 parts veal broth), and inject 1 c.c. of the broth culture subcutaneously into a guinea-pig. A control guinea-pig is injected with the same amount of the broth culture, and also a small quantity of antitoxin (0.5 c.c. of a 100 or 200 unit antitoxin, which cannot be used for other purposes). If the strain is a true diphtheria organism, the con-

* Received for publication June 30, 1915.

trol guinea-pig will live, while the test guinea-pig will die in from 2 to 3 days, showing the lesions typical of death from the effects of diphtheria toxin—subcutaneous edema, often extensive, at the site of injection, congested and hemorrhagic adrenals, fluid in the pleural cavities, and congestion of the lungs with areas of partial consolidation. This method is reliable, but an autopsy should be performed in all cases in order to exclude death from other causes.

Recently Neisser has suggested that the virulence of cultures be tested as follows:

> One loopful of a 24-hour Loeffler slant of the organism is suspended in each of 1 c.c., 10 c.c., and 100 c.c. of physiologic salt solution, and 0.1 c.c. of each suspension is injected intracutaneously into the abdominal surface of a guinea-pig. As a control, some antitoxin containing 8 units per cubic centimeter is added to an equal volume of the heaviest suspension, and 0.1 c.c. of the mixture is injected intracutaneously into the same guinea-pig. True virulent diphtheria bacilli will cause a characteristic local inflammatory lesion, with superficial necrosis, in from 48 to 72 hours, the intensity of the reaction depending upon the number of injected organisms and their virulence. The skin at the site of the control injection should remain normal in appearance. No lesions are produced when the bacillus xerosis or the bacillus hoffmanni, or the non-virulent diphtheria-like bacillus, is injected.

This method is analogous to that of Römer for the determination of small amounts of diphtheria antitoxin in sera. This consists of the intracutaneous injection of varying mixtures of the unknown antitoxic serum and a standard toxin; a slight excess of toxin produces a local necrosis which is in every way similar to that produced by virulent diphtheria bacilli, while a neutral or over-neutralized mixture shows no effect on the tissues at the site of injection.

The method recommended by Neisser is fairly satisfactory, but, following his directions, we have occasionally noted that the direct addition of antitoxin to the bacteria in the control injection immunized the animals sufficiently to affect the test lesions to a considerable degree. If the amount of antitoxin added is diminished to avoid this general immunization, the local action of the bacteria is not completely inhibited, so that lesions are found in both test and control areas.

For this reason the following modification of Neisser's method, which has been found to be both reliable and economical, is suggested.

METHOD

Two guinea-pigs, weighing about 350 grams, are used for the testing of from 4 to 6 different strains. One pig as a control receives 0.5 c.c. of antitoxin (about 500 units per cubic centimeter) intracardially at the time of the

tests, or intraperitoneally 24 hours before. The intracardial injection is the better, as it produces a complete inhibition of the local action of virulent bacteria in the control injections. The hair on the abdominal surface of each pig is removed, either by the application of a paste made of barium hydrosulphid, or preferably by pulling the hair out. This easily can be done, with less pain probably than is associated with the prolonged irritation following at times the application of the depilatory.

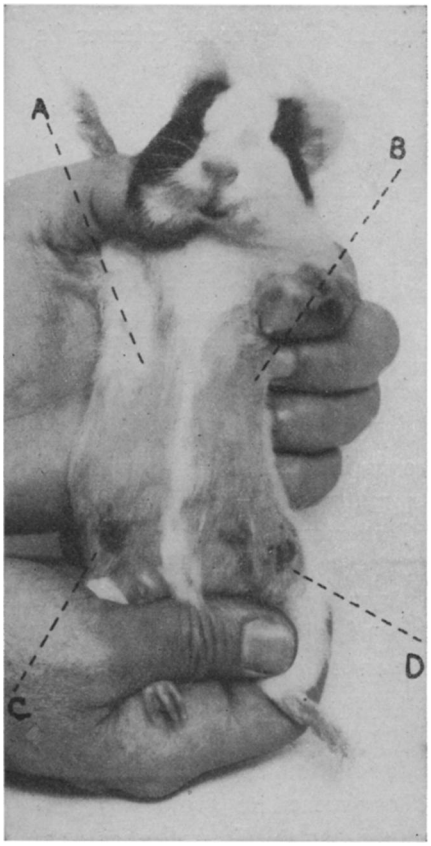

Figure 1

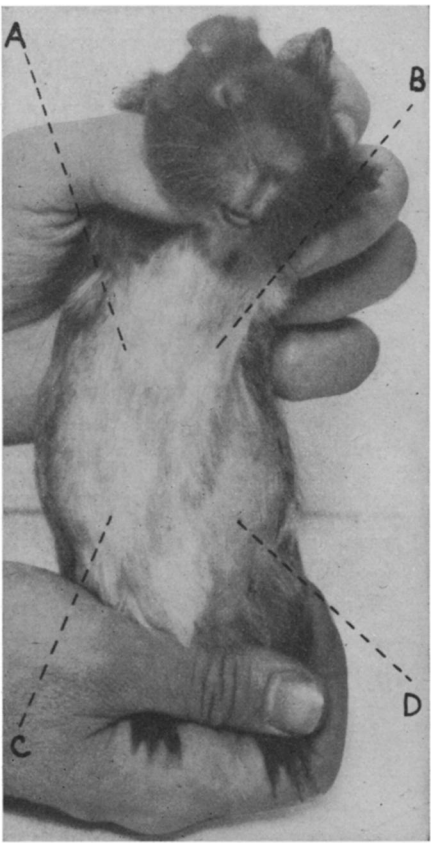

Figure 2

Fig. 1.—Test animal. This guinea-pig received intracutaneous injections, at points A, B, C, and D, of suspensions of 4 different strains of morphologically typical diphtheria-like bacilli. At A and B there are no lesions, an absence of virulence being thus indicated in these strains; at C and D there are distinct, circumscribed, indurated lesions with beginning superficial necrosis, showing that these two strains are virulent.

Fig. 2. — Control animal. This animal received 4 injections corresponding to those received by the guinea-pig in Figure 1, and, in addition, 0.5 c.c. of antitoxin intracardially. No lesions were produced at C and D; hence the specificity of the lesions shown in Figure 1 is proved.

For the bacterial emulsion, a fresh 24-hour growth from an ordinary Loeffler slant or a glucose ascitic agar slant of equal size, is suspended in 20 c.c. of normal salt solution. It is important that the growth be not more than 24 hours old, since many of the bacteria die if the culture is kept for 48 hours or longer in the thermostat. Ice-box preservation of grown cultures also kills many of the organisms. Loeffler slants are used similar to those furnished by the New York city department of health for the purpose of diagnosis in diphtheria. They should have a fairly uniform surface size and be sealed with paraffin to prevent drying of the medium. It is also important that the medium be not too acid, since excess of acid is apt to inhibit growth to a considerable degree.

Suspensions of the cultures to be tested having been prepared in the way described, 0.15 c.c. of each is injected intracutaneously into both guinea-pigs. The abdominal surface is divided into 4 or 6 areas, in accordance with the size of the guinea-pig, and the injections are made as far apart as possible in order to avoid a fusion of the lesions. Four strains can be tested out on a medium-sized guinea-pig, and 6 on a larger one, without the danger of overlapping. A 0.5 c.c. or 1 c.c. Record syringe with a very fine steel or platinum iridium needle is suitable for the injection. If the injections have been made properly, a circumscribed elevation appears, which persists for from 1 to 2 minutes.

The results of the tests are noted in 24, 48, and 72 hours. Virulent strains produce a definitely circumscribed, local inflammatory lesion, which shows a superficial necrosis in from 48 to 72 hours. In the control pig the skin remains normal, if the injections have been accurately carried out. With non-virulent strains, no lesion will be found in either control or test animal.

In this way 4 cultures can be tested on 2 animals, as compared with 8 animals necessary in the older way. The control animal may be used again within a week for another set of tests without a further injection of antitoxin. By the use of large guinea-pigs, on which 6 tests can be made, 10 or 12 can be saved, a considerable advantage when a large number of strains are to be tested.

In testing by this method 20 non-virulent and 40 virulent strains of diphtheria bacilli, we obtained results corresponding exactly with those of the usual and less economical subcutaneous test for virulence. This method is being used at present in the routine virulence testing at the research laboratory of the New York city department of health.